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Direct Sequencing and RipSeq Interpretation as a Tool for Identification of Polymicrobial Infections

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In this study, RipSeq Mixed, a software resolving uninterpretable mixed DNA sequencing chromatograms, revealed the bacterial content of 15 polymicrobial samples. Direct sequencing combined with RipSeq Mixed constitutes a valuable supplement to cultivation, particularly when cultivation is negative and direct sequencing is inconclusive despite continued clinical indications of infection.

In clinical microbiology, direct DNA Sanger sequencing based on broad-range PCR has become an important supplementary tool for fast identification of fastidious or attenuated microorganisms that cannot be found using standard cultivation techniques (1–5). The combination of broad-range PCR and direct sequencing is, however, incompatible with polymicrobial infections, resulting in uninterpretable mixed sequencing chromatograms. Consequently, polymicrobial infections might be reported as negative or inconclusive.

One way of resolving this is by separating the individual PCR products before sequencing by constructing clone libraries. This method is informative, but the laborious protocol makes it applicable for research purposes only and unsuitable as a routine diagnostic tool (6–8).

As a solution to the interpretation of chromatograms resulting from direct sequencing of polymicrobial samples, Isentio (Bergen, Norway) has introduced RipSeq Mixed for analysis of mixed chromatograms resulting from up to three different species (9). The software resolves the chromatograms and provides species names for the bacterial species present.

In a study conducted at Rigshospitalet (Copenhagen, Denmark), the bacterial contents of 293 culture-positive blood culture flasks from 176 patients were examined by direct sequencing of a 500-bp region of the 16S rRNA gene (10). For 15 samples, sequencing was inconclusive despite the fact that one or more species were identified by cultivation. The sequencing chromatograms of these 15 samples constituted a good demonstration case for evaluating the clinical potential of RipSeq Mixed. In this study, these initially inconclusive chromatograms were interpreted using RipSeq Mixed to see if this could help gain information from them. To be able to reveal DNA from more than three different species, nearly full-length 16S rRNA gene clone libraries were constructed for selected samples.

(Part of these data were presented as a poster [no. P1931] at the 21st European Congress of Clinical Microbiology and Infectious Diseases, Milan, Italy, May 2011.)

Blood was drawn to aerobic (10 ml) and anaerobic (10 ml) blood culture bottles (referred to as "ae" and "an," respectively) and cultivated for up to 7 days using the Bactec 9240 system (BD, Franklin Lakes, New Jersey). Culture-positive vials were subject to Gram staining and further colony identification as described previously (8). DNA was extracted from 100 μl culture-positive blood, following the procedure described by Kulski and Pryce (11). The first 500 bases of the bacterial 16S rRNA gene were amplified and sequenced using the MicroSeq 500 16S rDNA bacterial identification system according to the manufacturer's guidelines (Life Technologies, Paisley, United Kingdom), and inconclusive sequencing chromatograms were analyzed using RipSeq Mixed.

For selected samples, nearly full-length 16S rRNA gene sequences were amplified by 30 or 35 cycles of PCR using the 26F and 1390R primer pair (12). PCR and the insertion of PCR products into plasmids were carried out as described previously (8). Plasmids were amplified using the illustra TempliPhi kit (GE Healthcare, Waukesha, Wisconsin) and the inserts sequenced by Macrogen using the M13F and M13R primers. Similarity searches of the sequences were performed in the NCBI database with the BLAST tool excluding uncultured/environmental sample sequences from the reference sequences (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 1 summarizes the results obtained by Gram staining, cultivation, construction of clone libraries, and direct sequencing combined with RipSeq Mixed. Bacteria were identified in all 15 samples, of which 12 contained at least two different species, and for samples 1ae, 3an, 9ae, and 11ae (27%), there was complete correspondence between the cultivation data and the molecular methods. In seven cases, the number of identified species differed between the cultivation-based methods and the molecular techniques (for instance, samples 2ae, 7an, and 10an). These discrepancies might be explained by the biases associated with the different methods: PCR-based identification of microorganisms in polymicrobial samples is generally biased by various amplification efficiencies for different DNA sequences as well as the specificity of the applied primers (as an example, the use of primers targeting the 16S rRNA gene explains why the cultivation of *Candida albicans* in sample 8an was not confirmed by the molecular methods).

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TABLE 1 Patient information and microbiological findings by Gram staining, cultivation, and direct sequencing followed by RipSeq Mixed and in the clone libraries[a]

Sample	Patient information and antibiotic treatment at time of blood sampling	Gram staining	Cultivation	Direct sequencing followed by RipSeq Mixed	Clone library
1an	Male, 50 years old, abdominal surgery, anastomotic leak; cefuroxime and metronidazole administered	Gram-negative, nonmotile rods; Gram-positive cocci in chains	***Citrobacter freundii*, *Enterococcus faecalis***	***Citrobacter freundii*/*gillenii*/*youngae*/*Enterobacter asburiae*, *Enterococcus faecalis*, *Veillonella parvula***	31 clones: **5 *Citrobacter freundii*, 4 *Enterococcus faecalis***, 19 *Veillonella dispar*, 3 *Veillonella parvula*
1ae		Gram-negative, nonmotile rods; Gram-positive cocci in chains	***Citrobacter freundii*, *Enterococcus faecalis***	***Citrobacter freundii*/*gillenii*, *Enterococcus faecalis***	Clone library not constructed
2ae	Female, 72 years old, lymphoma, pneumonia, leukopenic; meropenem, ciprofloxacin, metronidazole, vancomycin, and caspofungin administered	Gram-positive cocci in chains; Gram-positive cocci in clusters	***Enterococcus faecium***, CoNS	***Enterococcus faecium***	32 clones: **32 *Enterococcus faecium***
3ae	Male, 62 years old. Ambustio. Similar culture results in several skin swabs. No antibiotics administered	Gram-negative, nonmotile rods; Gram-positive cocci in clusters	***Klebsiella oxytoca*, *Staphylococcus aureus***, *Micrococcus* sp.	*Enterobacter cloacae*/*ludwigii*/***Klebsiella oxytoca*, *Staphylococcus aureus***	18 clones: **2 *Klebsiella oxytoca*, 16 *Staphylococcus aureus***
3an		Gram-negative, nonmotile rods; Gram-positive cocci in clusters	***Klebsiella oxytoca*, *Staphylococcus aureus***	*Citrobacter youngae*/*Enterobacter cloacae*/*ludwigii*/***Klebsiella oxytoca*, *Staphylococcus aureus***	22 clones: **1 *Klebsiella oxytoca*, 21 *Staphylococcus aureus***
4ae	Male, 40 years old, chemical etch on 30% of the body, similar culture results in skin swabs; no antibiotics administered	Gram-positive rods; Gram-negative, motile rods	***Bacillus cereus*, *Enterobacter cloacae***, *Acinetobacter baumannii*, *Staphylococcus epidermidis*	***Bacillus cereus*/*anthracis*/*thuringiensis*, *Enterobacter cloacae*/*hormaechei*/*Escherichia coli*/*albertii*/*Shigella boydii*/*dysenteriae*/*sonnei*, *Kluyvera ascorbata***	23 clones: 6 *Bacillus* sp., **6 *Bacillus cereus***, 3 *Bacillus anthracis*, 4 *Enterobacter hormaechei*, **2 *Enterobacter cloacae***, 1 *Enterobacter* sp., 1 *Aeromonas veronii*
4an		Gram-negative, motile rods	***Enterobacter cloacae***, *Acinetobacter baumannii*	*Aeromonas hydrophila*, ***Enterobacter cloacae*/*hormaechei***	20 clones: 3 *Aeromonas veronii*, 5 *Aeromonas* sp., 9 *Enterobacter hormaechei*, **1 *Enterobacter cloacae***, 2 *Enterobacter* sp.
5an	Male, 26 years old, short bowel syndrome, tunneled CVC *in situ* for more than 1 year; no antibiotics administered	Gram-negative, motile rods; Gram-positive cocci in chains	***Escherichia coli***, *Klebsiella pneumoniae*, *Enterococcus faecalis*	*Veillonella dispar*/*parvula*, ***Escherichia coli*/***Shigella boydii***	37 clones: 9 *Veillonella* sp., 5 *Veillonella dispar*, 13 *Veillonella parvula*, **1 *Escherichia* sp.**, 1 *Enterococcus* sp.,1 *Enterococcus faecalis*, 6 *Clostridium clostridioforme*, 1 *Clostridium* sp.
6ae	Male, 49 years old, intravenous drug addict with short bowel syndrome, nearly new tunneled CVC, endocarditis considered but rebutted; no antibiotics administered	Gram-positive cocci in chains; Gram-positive cocci in clusters	***Streptococcus mitis***, *Gemella haemolysans*	***Streptococcus mitis***/genomosp. C1/*oralis*/*parasanguinis*/sp., *Streptococcus oligofermentans*, *Streptococcus* sp. (oral taxon 056)	39 clones: 10 *Streptococcus parasanguis*, 16 *Gemella haemolysans*, **8 *Streptococcus mitis***, 2 *Granulicatella adiacens*, 1 *Actinomyces* sp., 2 *Abiotrophia para-adiacens*
6an		Gram-positive cocci in chains; Gram-positive cocci in clusters	***Streptococcus mitis*, *Gemella haemolysans***, *Streptococcus salivarius*	***Streptococcus mitis***, *Abiotrophia para-adiacens*, ***Gemella haemolysans***	Clone library not constructed

7an	Male, 31 years old, Morbus Crohn, surgery for ileitis and re-operation due to leakage 1 month earlier; fluconazole administered	Gram-positive rods	***Propionibacterium acnes***	***Propionibacterium acnes*, *Staphylococcus aureus***	Clone library not constructed
8an	Female, 39 years old, short bowel syndrome, tunneled CVC probable focus; no antibiotics administered	Gram-positive cocci in clusters	***Staphylococcus epidermidis*, *Candida albicans***	***Staphylococcus epidermidis***	Clone library not constructed
9ae	Female, 62 years old, nephropathy, cardiomyopathy, affected skin, CVC; ciprofloxacin administered	Gram-positive cocci in chains	***Staphylococcus aureus*, Hemolytic streptococci grp. B**	***Staphylococcus aureus*, *Streptococcus agalactiae***	Clone library not constructed
10an	Male, 59 years old, malignant pancreatic tumor, changed stent in ductus; ampicillin administered	Gram-positive cocci in clusters; Gram-negative, nonmotile rods	***Klebsiella pneumoniae*, *Enterococcus faecium***	***Klebsiella pneumoniae***	Clone library not constructed
11ae	Male, 56 years old, hemodialysis, diabetes, poor dental status, endocarditis rebutted; no antibiotics administered	Gram-positive cocci in chains; Gram-positive cocci in clusters	***Streptococcus oralis*, CoNS**	***Streptococcus oralis*/*mitis*, *Staphylococcus epidermidis***	Clone library not constructed

[a] Species identified by both cultivation and molecular methods are in bold. The RipSeq algorithm excludes sequences with less than 99.3% identity to an organism in the database, and the results are listed according to descending score. For some samples, the algorithm was unable to distinguish between different species or subspecies. These are indicated with "/" for "and/or." For the clone libraries, only sequences with at least 99% identity to a BLAST hit in the NCBI database are included. "an" and "ae" indicate anaerobic or aerobic blood cultivation, respectively. CoNS, coagulase-negative staphylococci. CVC, central venous catheter.

Also, the efficiency of cell lysis and DNA extraction has a great impact on the outcome of PCR. Finally, very complex samples lead to more ambiguous positions in the chromatogram, which increases the number of possible sequences that the RipSeq algorithm can generate, including "false" ones. Cultivation, on the other hand, can be biased by administering antibiotics prior to blood sampling, growth conditions favoring some species over others, varying generation times for different species, or overgrowth of abundant species. For sample 1an, the facultative anaerobes *Citrobacter freundii* and *Enterococcus faecalis* were identified by anaerobic blood cultivation which corresponded well to the Gram staining of this sample. In addition to these species, DNA from the strict anaerobe *Veillonella parvula* was identified by RipSeq and in the clone library, but no Gram-negative cocci were observed in the sample. The fact that *Veillonella parvula* was not identified by culturing corresponds well with the patient being treated with metronidazole, an antibiotic especially effective against obligate anaerobic bacteria.

The RipSeq Web service is still relatively new and has only been described in a few published studies, where the results of direct sequencing and RipSeq analysis are compared to cultivation data (9, 13, 14). This study is the first comparing RipSeq to the construction of clone libraries, and overall, a good correlation was seen between the two molecular methods. However, in some cases, the clone libraries revealed DNA from additional species compared to RipSeq, for instance, samples 5an and 6ae. This might be a result of low abundance sequences "drowning" during direct sequencing, or it could be due to the fact that RipSeq can identify a maximum of three different species per sample, whereas for the clone libraries there is no upper limit.

Cultivation is a very central technique in the clinical microbiology, as it can provide a species identity and information about important phenotypic characteristics such as the antibiotic susceptibility. However, PCR-based methods can be a valuable supplement to the traditional techniques for identification of nonculturable or inactive microorganisms or when a rapid identification is crucial.

Direct sequencing is particularly useful for samples obtained from sterile body sites, and at the Department of Clinical Microbiology at Rigshospitalet, it is routinely considered for use on such culture-negative samples if infection is still suspected (e.g., for spinal fluids, heart valves in infectious endocarditis, or brain abscesses). In this study, information was gained from 15 inconclusive sequencing chromatograms using RipSeq Mixed. This shows the value of the software for clinical microbiological departments, where direct sequencing is established as a routine technique—it facilitates the application of direct sequencing to specimens that are typically polymicrobial.

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